ORIGINAL ARTICLE

The Effects of Hydroxyapatite-Gelatin-Propolis Elution on Osteoblast Cell Culture

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ABSTRACT

Introduction: Hydroxyapatite (HA) and gelatin and propolis materials have been investigated and combined to enhance bone regeneration. This study aimed to evaluate the levels of total protein, protein profile, alkaline phosphatase (ALP) and osteocalcin (OC) in the culture medium of osteoblast cells following exposure to HA, gelatin, and propolis elution. Materials and Methods: Human Osteoblast Cell-line MG-63 was cultured in six exposure groups, including control, HA, 6% propolis, HA-gelatin, HA-6% propolis, and HA-gelatin-6% propolis. ALP and OC levels were analyzed and quantified in the culture medium after 7, 14, and 21 days of exposure using the ELISA test. Then the Bradford test was performed to see the total protein and SDS-PAGE test to see the protein profile. Results: Higher levels of total protein were observed in the HA group, HA-6% propolis group, and the 6% propolis group compared to the control group. Conversely, no differences were noted in the protein profiles among all exposure groups. The HA group, 6% propolis, and HA-gelatin exhibited higher levels of ALP and OC levels compared to the control group. ALP levels were increase positively correlated with an increase in OC levels in all group. Conclusions: There was no significant difference in the total protein, protein profile, levels of alkaline phosphatase and osteocalcin in the hydroxyapatite-gelatin-propolis 6% elution exposure group compared to the control group. However, the results of the study showed that there was an increase in the total protein, ALP level, and OC level of the osteoblast cell culture following the administration propolis.
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INTRODUCTION

Bone is a dynamic tissue that renews its tissue continuously through the process of bone remodeling. Bone remodeling relies on a balance of bone resorption through osteoclasts with bone deposition through osteoblasts (1). However, excessive resorption can occur and cause bone loss as is found in several diseases, for example in post-menopausal osteoporosis, rheumatoid arthritis, and periodontitis (2). In the alveolar bone, bone loss or bone defects can occur in the maxilla and mandible due to various causes, such as trauma, bone deficiency following tumor resection, congenital anomalies, periodontal disease, and tooth loss (3). Complicated and extensive bone damage can inhibit remodeling and healing (4). Therefore, understanding the molecular and cellular mechanisms which trigger osteoblast formation is vital to provide alternative treatments for the conditions above and other conditions. (5).

Bone is composed of a mineral phase (hydroxyapatite), an organic phase (90% collagen type 1, 5 % non-collagen protein, and 2% lipid), and water (6). Therefore, bone tissue engineering has been developed using materials whose structure resembles bone. Bone tissue engineering aims to trigger the regeneration of new and functional bone with a mix of biomaterials, cells, and growth factor therapy (7).

One of the bone tissue engineering materials that is widely used is HA which has similarities to the bone mineral phase and has been proven to be biocompatible,

osteoconductive, and also bioactive with native bone tissue (8-12). Despite its advantages, HA is brittle. The compressive strength of HA becomes low due to its high level of porosity. This phenomenon causes HA to easily break when subjected to both small and large forces. Therefore, HA is strengthened by the addition of polymers, which are generally chitosan or gelatin. Gelatin is a natural polymer obtained from collagen hydrolysis, which has elastic properties. Gelatin is a scaffold material that is widely used because it is relatively cheap (13). Various literature has discussed the combination of HA-gelatin as a biomaterial using various fabrication methods (14-16).

In this study, the author wants to know the impact of introducing propolis to HA-gelatin given propolis' extensive utilization within the field of dentistry. Propolis has various bioactive components in the form of terpenoids, quercetin, saponin, cinnamic acid, flavonoids, apigenin, artepyline, and caffeic acid-phenetyl ester (CAPE) which has various helpful effects, including antioxidant, antiviral, anti-inflammatory, antifungal, antibacterial, anticancer, and immunostimulative (17). CAPE can stimulate tissue growth and elevate bone growth biomarkers. Osteoblast cells are specialized bone formation cells that are regulated by several growth factors, in the contents of propolis in the form of CAPE, flavonoids, saponins, cinnamic acid, and quercetin each have the ability to influence proteins that regulate osteoblast activity, thereby triggering bone remodeling (17).

This study analyzes the total protein, protein profile, ALP and OC levels in osteoblast cell culture following exposure of hydroxyapatite, gelatin and propolis to see its effect on osteoblast cell activity. Proteins are cell components that play an important part in cell activities, such as cell growth, division, and differentiation. Total protein in cell culture medium can be used as an indicator of cell response to a substance or biomaterial. Meanwhile, the protein profile is a protein expressed by tissue under certain conditions or time and is used to evaluate variable additions to the pattern of proteins secreted by osteoblast cells (18). The assessment of bone remodeling processes can be observed through bone formation markers. Metabolic products commonly used in assessing osteoblast activity during bone regeneration processes are alkaline phosphatase (ALP) and osteocalcin (OC) (19). ALP is an enzyme secreted by osteoblasts. Generally, ALP is considered an early marker of osteoblast differentiation and mineralization (20). Osteocalcin is the most abundant non-collagenous protein produced by osteoblasts, with a high affinity for calcium and playing a crucial role in mineralization and calcium ion homeostasis processes. Biomarkers such as alkaline phosphatase and osteocalcin can be utilized to observe the effects of therapeutic agents or the osteoconductive properties of materials on bone (21).

MATERIALS AND METHODS

This research was conducted as an in-vitro laboratory experimental study and aim to assess the levels of total protein, protein profile, alkaline phosphatase (ALP) and osteocalcin (OC) in the culture medium of osteoblast cells following exposure to a hydroxyapatite, gelatin, and propolis elution. Human Osteoblast Cell-line MG-63 (ATTC No. CRL-1427) was cultured in six exposure groups, including control, HA, 6% propolis, HA-gelatin, HA-propolis 6%, and HA-gelatin-propolis 6%. After 7, 14, and 21 days of exposure, ALP and OC levels were analyzed and quantified in the culture medium using the ELISA test. The total protein was performed using Bradford test and SDS-PAGE test to see the protein profile.

MG-63 cells were transferred into a 15 ml tube pre-filled with 9 ml of DMEM medium supplemented with 10% FBS and 3% antibiotics. Following this, the cells were centrifuged at 2000 rpm for 5 minutes. After discarding the supernatant, fresh medium was added for subsequent resuspension of the cells. The cells were then transferred into a culture flask containing 4 mL of DMEM medium supplemented with 10% FBS and 3% antibiotics. Cell cultures were incubated in a CO_2 incubator at 37°C with 5% CO_2. The culture medium was changed every 2 days, and cell growth ratio and confluency were observed under a microscope. Cells were incubated in a 37°C incubator for 2-5 minutes. Cells were then harvested and seeded at 1 × 105 cells per well.

The preparation of the elution carried out with percentage of HA, gelatin, and propolis in the saline solvent used in this study was 7.5%w/v, with HA comprising 30% of the total material percentage. The preparation of elution was initiated by dissolving gelatin in saline using a beaker glass according to the concentration on a hot plate at a temperature of 40°C and stirred using a glass rod stirrer. Subsequently, HA and propolis were added at predetermined concentrations, with continuous stirring using a magnetic stirrer. The formed elution was then transferred into 15 ml tubes and vortexed on a Vortex Mixer to mix the elution. The tubes containing elution were centrifuged for 5 minutes at a speed of 2000 rpm, followed by filtration of the elution using a 20-25 mikron microfilter before being transferred into new tubes.

The cell culture medium was then discarded and replaced with 100 µL of complete medium which has been added with 10 µL of exposure material in 96 well plates and 1000 µL which has been added with 100 µL of exposure material in 24 well plates. After exposure, the cells were incubated again in an incubator under conditions of 5% CO_2 at 37°C. The cell culture medium was replaced every 3 days. ELISA, the Bradford test and SDS-PAGE were used for later analysis.

The data obtained consisted of numeric descriptive

data, which were analyzed using the Statistical Programme for Social Science (SPSS) version 25 with a 95% confidence interval. Data processing begin with frequency distribution analysis and data normality testing with the Shapiro-Wilk test. Next, a comparative analysis test was carried out to see the comparison of alkaline phosphatase and osteocalcin levels for each treatment group. If the data is normally distributed, the 1-Way Analysis of Variance (ANOVA) test will be used, whereas if the data is not normally distributed, the Kruskal Wallis Test will be used. A correlation test was also carried out to see the relationship between alkaline phosphatase and osteocalcin levels. If the data is normally distributed, the Pearson correlation test is used, whereas if the data is not normally distributed, the Spearman correlation test is used. Data are considered statistically significantly different if the p-value is less than 0.05 ($p<0.05$).

RESULT

Total Protein Concentration Measurement Results in Figure 1. Shown here the total protein in the osteoblast cell culture medium given hydroxyapatite-gelatin-6% propolis elution and the control group as mentioned before.

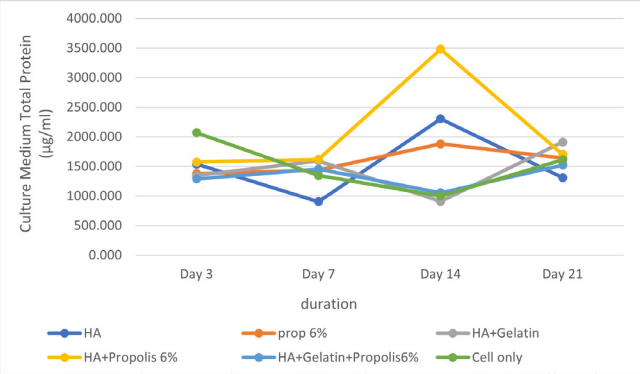

Figure 1: Graph of Total Protein Levels of Osteoblast Cell Culture Medium with the Administration of Hydroxyapatite-Gelatin-Propolis 6% Elution Compared to the Control Group on days 3, 7, 14, and 21 after Exposure.

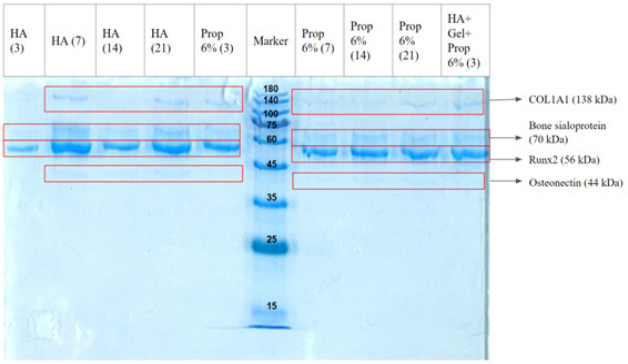

Figure 2: SDS-PAGE test

In the SDS-PAGE test results, several protein masses were found as shown in Figure 2. The protein mass between the 100 kDa and 140 kDa ladders were found in every sample except day 3 HA. Then the protein between the 60 kDa and 75 kDa ladders were found in all samples. Proteins mass between the 45 kDa and 60 kDa ladders were also found in all samples. The protein mass under the 45 kDa ladder were found in every sample except day 3 HA.

Figure 3 showed that alkaline phosphatase levels in each exposure and control group had the same tendency, increasing from the 7th to 14th day and starting to decrease on the 21st day. The highest peak of alkaline phosphatase levels was found on the 14th day. The outcome of the descriptive analysis showed that the HA, 6% propolis group, and the HA-gelatin group had higher alkaline phosphatase levels compared to the control group (cell only), while the 6% propolis hydroxyapatite-gelatin group had lower levels than the control group. Statistical tests to see significant differences between groups in each day of exposure test were carried out using the Kruskal Wallis Test and followed by the Mann-Whitney Post Hoc Test. There were no statistically significant difference in ALP levels between the HA-gelatin-6% propolis group and the control group on all test days. However, there was a statistically significant difference ($p<0.05$) in ALP levels between 6% propolis group and control group on the 14th day ($p =0.018$) .

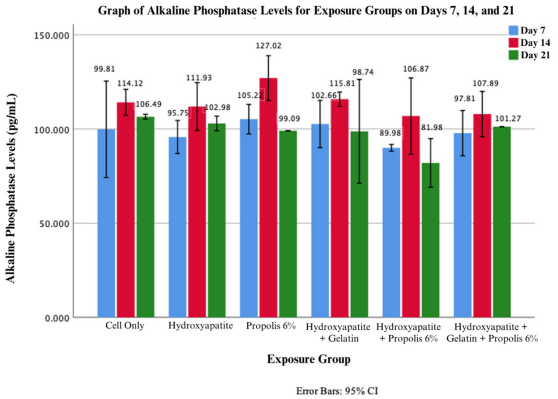

Figure 3: Graph of Alkaline Phosphatase Levels

Figure 4 showed that osteocalcin levels in each exposure and control group had the same tendency, increasing from the 7th to 14th day and starting to decrease on the 21st day. The results of the descriptive analysis showed that the HA group, 6% propolis group, and the HA-gelatin group had higher osteocalcin levels compared to the control group (cell only). Statistical tests to see significant differences between groups in each day of exposure test were carried out using the Kruskal Wallis Test and followed by the Mann-Whitney Post Hoc Test. There were no statistically significant difference in OC levels between the HA-gelatin-6% propolis group and the control group on all test days. However, there was a statistically significant difference ($p<0.05$) in OC levels between 6% propolis group and control group on days 14 ($p = 0.018$) and 21 ($p = 0.018$).

Based on the Spearman statistical test, correlation analysis showed a statistically significant moderate positive linear relationship ($r = 0.385$, $p = 0.001$)

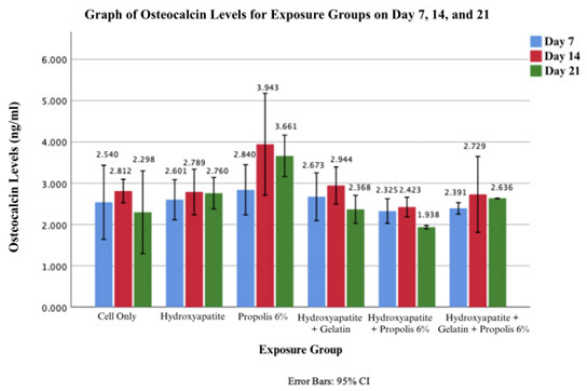

Figure 4: Graph of Osteocalcin Levels

between alkaline phosphatase levels and osteocalcin, higher alkaline phosphatase levels correlated with higher osteocalcin levels as seen in Table I.

Table I: Correlation analysis showed higher alkaline phosphatase levels correlated with higher osteocalcin levels.

	r Value	p Value
Alkaline Phosphatase		
	0,385	0,001*
Osteocalcin		

DISCUSSION

Differences in total protein levels were observed across all the groups, and several profile proteins detected. In the HA, HA-propolis 6%, and propolis 6% groups, there was an increase in concentration that peaked on the 14th day. The total protein concentration in the HA-propolis 6% group was the highest compared to the other groups. This indicates that propolis can enhance the proliferation and maturation of osteoblast cells, as demonstrated in previous studies. The HA-gelatin and HA-gelatin-propolis 6% groups exhibited similar concentration levels, with a slight increase observed on the 7th day. Subsequently, both groups experienced a decline on the 14th day. The decrease until the 14th day is suspected to be a consequence of gelatin material requiring specific methods to bind with HA in solution. However, no differences were noted in the protein profiles among all exposure groups. This study still required further investigation to identify specific protein profiles with specific antibodies using Western blot analysis method.

The HA group, the 6% propolis group, and the HA-gelatin group exhibited higher levels of ALP and OC in comparison to the control group. Moreover, there was a positive correlation between the increase in ALP levels and the increase in OC levels. ALP plays a crucial role in calcium and phosphate deposition into the bone matrix (22) Based on the literature, osteoblasts experience a period of proliferation in the first 7 days, it is during this period that alkaline phosphatase levels begin to appear (23). In this study the ALP levels of all exposure groups were visible on the 7th day, reach the peak on 14th day, and decreased on 21th day. The outcome of these findings are also in line with research by Lim, et al (2019) which showed that alkaline phosphatase levels in the osteoblast differentiation process in the initial markers of the bone regeneration process in vitro experienced peak levels on day 14 (24). Research by Shen, et al (2009) which evaluated osteoblast cell differentiation by looking at alkaline phosphatase expression on days 14 and 21, also showed results of an increase in alkaline phosphatase expression on day 14 and then a decrease on day 21 (25). In other literature, ALP levels were reported to increase in the initial stages of osteoblast differentiation, which peaks around the 15th-16th day. After that, ALP expression will decrease slowly, suggesting that there has been maturation and upregulation of several subsequent biomarker expressions such as Osteopontin (OPN), an extracellular structural protein responsible for ECM mineralization (26, 27).

The 6% propolis group showed a statistically significant difference ($p<0.05$) to the control group on the 14th day. This falls in line with previous research conducted by Lim, et al (2019) on the Human osteoblast-like cell line MG-63 where alkaline phosphatase activity escalated from the 7th to 14th day in all treatment groups given propolis extract compared to the negative control ($p<0.05$) (25). The increase in alkaline phosphatase levels in this group occurred because propolis contains polyphenolic compounds such as flavonoids and caffeic acid phenethyl ester (CAPE) which can increase osteoblast cell activity and increase the number of biomarkers or markers of bone formation such as ALP. Propolis extract can hasten osteoblast cell maturation and bone remodeling activity by enhancing osterix and Runx2. The saponins contained in propolis also play an active role in intensifying alkaline phosphatase activity, increasing mineralization and encouraging the expression of the osteogenic gene ALP and the Runx2 gene. Runx2 itself is a transcription factor involved with osteoblast differentiation and bone formation. It has been found that RUNX2 can prompt differentiation of mesenchymal stem cells into preosteoblasts and block differentiation into adipocytes. Cinnamic acid contained in propolis apart from acting as an immunomodulator, can also raise ALP and calcium activity which can stimulate bone formation while inhibiting the production of Nf-KB and TNF-α (29).

Based on previous research conducted by Vozzi et al (2014), collagen-gelatin- genipin-hydroxyapatite testing was able to increase ALP activity from day 3 to day 21 at 10%, 20%, and 30% (24). Results of Sarkar's research, et al (2018) also showed a fairly high increase

in alkaline phosphatase activity in human osteoblast cell line MG-63 cells on day 14 in the test group containing hydroxyapatite-gelatin composite. The increase in this group compared to the control group and the hydroxyapatite alone group was due to the structure and strong bond affinity between the two materials increasing cell attachment and growth. The combination of these two materials can improve the mechanical and thermal properties of hydroxyapatite-gelatin, and can increase the differentiation ability, especially if developed in the form of a composite/scaffold (26).

Osteocalcin takes part in bone mineralization and calcium ion homeostasis. In the maturation phase, osteoblasts will produce osteocalcin which will bind calcium and hydroxyapatite and help the nucleation process of mineralization of the bone matrix. This is in line with Beck et al's research where osteocalcin expression correlated with the transition to the mineralization phase and osteocalcin levels reached a peak around day 14 (23).

There was no statistically significant difference ($p>0.05$) in osteocalcin levels exposed to hydroxyapatite-gelatin-6% propolis in comparison to the control group on all test days. The increase in osteocalcin levels in comparison to the control group that occurred on day 21 could not be explained specifically, the condition was caused by the macroscopic condition of the cells looked unstable and experienced early apoptotic changes in the test group.

Just like alkaline phosphatase levels, the flavonoid and CAPE content can also accelerate osteointegration which is in line with the expression of osteocalcin as a marker/biomarker of bone formation (30). In this study the OC levels of all exposure groups were visible on the 7th day, reach the peak on 14th day, and decreased on 21th day. Previous research by Lim, et al (2019) on the Human osteoblast-like cell line MG-63 also showed that the treatment group given propolis extract experienced an increase in osteocalcin levels compared to the group without treatment from the first day to the end of the study on day 8 (26). Hydroxyapatite can support revascularization as well as adhesion, growth, differentiation of osteprogenitor cells and osteoblasts (10). Osteocalcin levels in the hydroxyapatite-gelatin group were above those in the hydroxyapatite alone group. This falls in line with research by Vozzi et al (2014) where the combination of collagen and hydroxyapatite in collagen-gelatin-genipin-hydroxyapatite was also able to increase osteocalcin activity from day 3 to day 21 at Hap 10%, 20%, and 30% better than the hydroxyapatite alone group (26).

The absence of an increase and statistically significant difference in both alkaline phosphatase and osteocalcin levels in the main test group given hydroxyapatite-gelatin-6% propolis elution has never been described in previous research or literature. This possibility could be caused by the instability of gelatin when combined with various other materials, especially those that have never been tested before. Based on research by Bello, et al (2020) for biomaterial purposes, gelatin-based materials have other main weaknesses, namely less stability in solution and a relatively briefer degradation rate when combined with various additional ingredients. When used in research requiring longer periods of time such as wound healing, cell differentiation, and controlled drug release, gelatin-based materials may not last as long (29, 31).

Crosslinking materials can function to increase the mechanical stability of materials when combined with other materials, increasing biocompatibility, bioactivity, and to overcome rapid degradation (29, 31-32). Based on research, Vozzi, et al (2014) combination of hydroxyapatite and gelatin and the addition of other materials with cross-linking. The linker does not interfere and is biocompatible with the growth of osteoblast cells. MG-63 can even increase the porosity of the scaffold (26).

This study also aims to determine the correlation between alkaline phosphatase and osteocalcin levels in the test group. Statistical analysis of Spearman's correlation showed a result that there is a moderate positive linear correlation (correlation) that is statistically significant between alkaline phosphatase and osteocalcin levels, where the higher the alkaline phosphatase level, the higher the osteocalcin level. This conclusion falls in line with research by Havill, et al which showed that levels of alkaline phosphatase and osteocalcin as markers of bone growth were positively correlated with each other ($r = 0.3844$, $p < 0.001$) with alkaline phosphatase being expressed earlier than osteocalcin (26, 33-34). This statistically significant positive correlation is because alkaline phosphatase and osteocalcin are molecular markers that can be detected well in osteoblasts during differentiation (22).

Combination of hydroxyapatite-gelatin-6% propolis elution in this study did not increase the levels of alkaline phosphatase and osteocalcin in osteoblast cells. However, the groups exposed to hydroxyapatite, hydroxyapatite-gelatin, and 6% propolis experienced an increase in alkaline phosphatase and osteocalcin levels. Considering this potential, further research and experimentation are needed in the future to explore the combination of these promising materials into composites or three-dimensional porous scaffolds with cross-linkers to produce more stable material combinations. The combination is also able to provide 3-dimensional space for cells to survive, increase their number, and to help cells in obtaining nutrients, especially useful in forming new bone tissue for healing bone defects (32).

CONCLUSION

There was no significant difference in the total protein, protein profile, levels of alkaline phosphatase and osteocalcin in the hydroxyapatite-gelatin-propolis 6% elution exposure group compared to the control group. Thus, the exposure to hydroxyapatite-gelatin-propolis 6% elution in the study did not affect the activity of MG-63 osteoblast cells, as observed from the protein and biomarkers produced. However, the results of the study showed that there was an increase in the total protein, ALP level, and OC level of the osteoblast cell culture following the administration propolis. This study also showed the higher the alkaline phosphatase level, the higher the osteocalcin level.

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